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1 Coagulation status in dogs with naturally occurring Angiostrongylus vasorum
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Abstract

Angiostrongylus is associated with bleeding tendencies in approximately 1/3 of clinical cases, but the cause of the coagulopathy is poorly understood although DIC is a proposed cause (Koch & Willesen 2009). Thromboelastography (TEG) is a global evaluation of coagulation and has not been described in a cohort of dogs with this disease. Thromboelastography in association with other measures of coagulation, PT, aPTT, antithrombin percentage activity, d-dimer concentration and von Willebrand factor concentration were evaluated in a group of 30 dogs with *A.varosum* infection. 18 dogs had signs of bleeding on physical examination. TEG was consistent with hypocoagulation in 17 of these dogs. There was no association between any of the other measures and hypocoagulation on TEG. Abnormal coagulation times were not significantly associated with the presence of bleeding. Only fibrinogen concentration was significantly different, (lower) in dogs that were bleeding compared to those that weren't. D-dimer concentrations were increased in 22/25 cases in the study, however other coagulation parameters were more variable. Although the changes identified in this study were not consistent within groups there is activation of coagulation within this population, possibly consistent with an intravascular disseminated coagulopathy.

Introduction

Naturally occurring *Angiostrongylus vasorum* infection is associated with a number of clinical syndromes in dogs. Bleeding tendencies were recognized in approximately 1/3 of cases diagnosed in England in a small case series (Chapman *et al* 2004).While the coagulopathy associated with *A.vasorum* has been clinically recognized for some time the published information is limited to case reports and experimental work (Schelling *et al* 1986, Ramsey *et al* 1996, Gould & McInnes 1999, Cury *et al* 2002 and Whitley *et al* 2005). A recent paper suggests that the parasite in South America may be genetically distinct from the parasite associated with natural infection in Europe throwing the clinical

relevance of some of this experimental work to European patients into question (Jefferies *et al* 2009).

There are a number of theories postulated that could explain the pathogenesis of the bleeding diatheses seen in these patients, of which the presence of chronic disseminated intravascular coagulation (DIC) seems to be most widely accepted (Koch & Willesen 2009). Other theories include acquired deficits in von Willebrands factor, accumulation of immune complexes stimulating the intrinsic coagulation system, immune mediated thrombocytopenia or inhibition of coagulation due to factors secreted by the parasite (Caruso & Prestwood 1988, Ramsey *et al* 1996, Gould & McInnes 1999, and Whitley *et al* 2005, O'Neill *et al* 2010). These studies have all used traditional methods of laboratory assessment of haemostasis. Thromboelastography is a newer technique that provides a global assessment of coagulation. It assesses both the influence of primary and secondary coagulation on blood clotting, and can also be used to identify hypercoagulability and clot strength. Thromboelastography in dogs with DIC has been previously performed, with the most common finding being evidence of hypercoagulability (Wiinberg B *et al* 2008). In this study 5 dogs had *A. vasorum* infection, 4 of which were hypercoagulable with the fifth normal on thromboelastography. It was not reported whether these dogs had signs of bleeding or not.

This study describes the haemostatic abnormalities seen in dogs diagnosed with naturally occurring *A.vasorum* in an endemic area of England through evaluation of traditional and global coagulation tests including thromboelastography.

Further evaluation for the presence of thrombosis and fibrinolysis was also performed looking for markers of DIC such as increased D-dimers and low fibrinogen concentrations. As thromboelastography (TEG) is thought to be a more global assessment for bleeding tendancies than PT and PTT we theorized that all dogs with signs of haemorrhage would have abnormalities on TEG. We also theorized that dogs presenting with clinical signs of haemorrhage would have more marked abnormalities in their coagulation parameters than those that were not confirmed to be bleeding.

Materials and methods

Dogs presenting at a referral hospital with a diagnosis of *Angiostrongylus vasorum* were enrolled into the study. Dogs were referred to the hospital for investigation and management of a variety of clinical signs and were diagnosed with the condition during investigation. Diagnosis of *A.vasorum* was based upon identification of L3 larvae in the faeces of affected dogs using Baermann sediment evaluation. Faecal samples were collected by collection after voiding or rectal examination. In most cases a single faecal sample was used for Baermann examination due to the requirement for a rapid diagnosis. As the result of this diagnosis is often delayed by 24 hours and dogs were enrolled if suspicion of the disease was high and later excluded if Baermann was negative. Dogs were also excluded if treated with agents effective against *A.vasorum* prior to testing. Owner consent was obtained for enrollment in the study. Institutional ethical approval was obtained prior to the start of the study.

Clinical information collected for each case included signalment, main presenting signs, whether there were bleeding diatheses on physical examination, post-mortem examination or magnetic resonance imaging and outcome.

Blood was collected atraumatically by venipuncture using a syringe and needle of the jugular or saphenous vein and submitted for analysis on the day of admission.

Thromboelastography was performed between 30 minutes and 2 hours after sampling according to in-house standard operating procedures. The samples were not activated prior to analysis. Citrated whole blood samples were used and added to a cup containing 280mmol $CaCl_2$ to give a total volume of 360ul/cup. A heparinase cup was used in all samples in case of prior heparin exposure. The analyses were run for a total of 120 minutes and readings were obtained continuously by the machine during this time. Measurements obtained from the analysis included reaction time (R), Clotting time (K), Angle (alpha), Maximum amplitude (MA) and global clot strength (G). Results were compared with a previously established reference range for this machine, using this technique (Goodwin *et al* 2011).

Excess citrated plasma was obtained by centrifugation at 4000x g for 2 minutes; it was then separated and frozen at -30°C until analysis via batch submission to Cornell diagnostic laboratories. Samples were sent frozen on ice within 3 months of sampling. The coagulation profile included prothrombin time (PT), activated partial thromboplastin time (aPTT), antithrombin percentage activity (AT) by chromogenic substrate method, fibrinogen concentration using the Clauss method, von Willebrand's factor antigen concentration by ELISA and d-dimer concentration by quantitative immunoturbidometric assay. The reference intervals were provided by the laboratory performing the analysis. Automated platelet counts were performed on EDTA blood and a minimum platelet count estimate was performed on blood smear examination by a clinical pathologist. If platelet counts could not be estimated due to clumping the clinical pathologist made a rough assessment based upon a count in the body of the smear and this was defined as adequate if greater than $150x10^9$/l.

Dogs were defined as having increases of PT and aPTT if they were greater than 125% of the top end of the reference interval. Dogs were defined as being hypocoagulable if they had 2 or more of 4 of the following; increased R, increased k, decreased alpha or decreased MA and hypercoagulable if 2 or more of the following were present; decreased R, decreased k, increased alpha or increased MA. Decreased G was used as a global measure of hypocoagulability and increased G as a global measure of hypercoagulability.

Statistical analysis

Data were entered into a statistical package for analysis[A]. Data were analysed for normality graphically and using the Kolmogorov-Smirnov test and are presented as mean (+/- s.d.) when normally distributed and median (range) when not. For analysis the data were categorized into dogs with clinical signs of bleeding and those that did not. Mann-Whitney U test was used for comparison of groups in non-parametric data and an independent samples T-test was used for parametric data. A Fishers exact data was used for categorical data. A p value of less than 0.05 was considered significant.

Results

A total of 30 dogs were enrolled into the study. All dogs had a confirmed diagnosis of *A.vasorum* using the Baermann method after initial enrollment. Complete data was available for 25 cases. In 5 cases thromboelastography was performed but traditional coagulation data was unavailable. The majority of dogs were purebred and represented eighteen different breeds with Staffordshire bull terrier (6) and cocker spaniel (4) most commonly represented. There were 8 female entire, 8 female neutered, 10 male entire and 4 male neutered. Median age was 2 years (range 0.3- 11). Presenting signs included neurological abnormalities in 16 dogs, including seizures, ataxia, coma and paralysis; spontaneous non-traumatic bleeding in 10, dyspnoea in 9 and polydipsia and polyuria related to hypercalcaemia in 2. Some dogs presented with more than one sign, most commonly neurological signs and bleeding into the sclera.

Eighteen dogs presented with bleeding diatheses on physical examination, imaging or at post-mortem examination. In a further 3 dogs bleeding was suspected in the central nervous system, but not confirmed as no imaging or investigation was performed; for statistical analysis these dogs are included in the not bleeding group. In 9 dogs no bleeding was identified or suspected. Twenty-one dogs survived to discharge. Of the nine dogs that died 6 had bleeding signs on presentation.

Platelet counts were available in 18 dogs. Minimum estimates were made by a clinical pathologist from a smear examination. Clumps were present in 8 of these blood smears making accurate estimation impossible. In a further 3 dogs the platelet count was stated to be adequate. Thrombocytopaenia (platelet count <150 without clumps present) was confirmed in 4 dogs. The median (range) platelet count using the minimum estimated count was 133 $x10^9$/L (40-342). Due to the subjective nature of this data no further analysis has been performed.

The coagulation panel was measured in 25 dogs. Fifteen of these had signs of bleeding. Prothrombin time and aPTT were prolonged in 8/25, aPTT was abnormal in a further 4/25 cases. Fibrinogen was decreased in 9/18 and increased in 9/18 cases. Antithrombin was within reference interval in 24/25

cases (median 92.5% range 45-119 reference interval 65-145) and decreased in 1/25. D-dimer concentrations were increased in 22/25 cases, (median 611ng/ml range 50-1511, reference interval 0-250). Von Willebrand's factor antigen concentration was decreased in 4/25 cases and increased in 8/25 cases. At least one coagulation measure was abnormal in 24/25 dogs and 3 or more were abnormal in 14 dogs.

Nine out of ten dogs that did not have evidence of bleeding had normal coagulation times (PT and aPTT). Eight out of 15 dogs with bleeding diatheses had normal coagulation times. Abnormal coagulation times were not significantly associated with the presence of bleeding diatheses.

There was no difference between antithrombin percentage activity, d-dimer concentration and von Willebrand factor antigen concentrations in dogs that were bleeding and those that were not bleeding. Fibrinogen concentration was lower in those that were bleeding (median 108mg/dL, range 15-895), compared with those that were not (median 545mg/dL, range 45-885) (p=0.026). Table 1 summarises these findings. Fibrinogen concentration was also lower in dogs that had abnormal coagulation times (median 40,mg/dL range 15-50), compared with those that did not (median 544mg/dL, range 108-895) (p=0.001).

TEG analysis was performed in 30 dogs. Hypocoagulability was present in 22 out of 30 dogs. Seventeen of the 18 dogs with bleeding signs were hypocoagulable on TEG. Five dogs with no evidence of bleeding were hypocoagulable on TEG, two of these dogs had neurological signs; seizures and hind limb paralysis, with the remainder presenting for dyspnoea. Hypocoagulability identified on TEG was associated with the presence bleeding diatheses (p=0.003). Hypercoagulability was present in 3 out of 30 dogs. None of these 3 dogs had signs of bleeding. Two of these dogs were diagnosed with pulmonary hypertension. No other dogs were diagnosed with pulmonary hypertension in this study, however echocardiography was not performed in all cases. There were significant differences identified in R-time (R) (p=0.008), angle (a) (p=0.001), maximum amplitude (MA) (p=0.004) and G (p=0.004) between dogs that were bleeding and those that were not bleeding (Table 2). K

time could not be analysed as in 12 dogs the clot did not reach sufficient strength to be able to allow measurement of a k time. There was no difference in fibrinogen concentrations, d-Dimer concentration, antithrombin percentage activity or von Willebrand factor concentration between dogs that were hypocoagulable on TEG and those that were not (table 3).

Outcome was not different between dogs that had bleeding diatheses, had abnormal PT or aPTT, or were hypocoagulable on TEG analysis.

Discussion

This is the first study to describe some of the changes in coagulation in a large population of naturally infected dogs presenting with clinical signs of angiostrongylosis including patients both with and without bleeding diatheses. It is also the first study to report on TEG findings in this patient group. It should be noted that the population was a referral population and therefore likely represents a more severely affected population than seen in first opinion practice; the changes reported therefore may not represent the changes seen in less severely affected dogs. The incidence of bleeding in first opinion cases is likely to be lower, as seen in the study by Willesen *et al* (2009).

The signalment of dogs in this study is similar to that in other clinical studies (Chapman *et al* 2004, Willesen *et al* 2009) although the incidence of bleeding signs is higher in the group reported here. This is likely to be related to the increased awareness of the disease over the last 10 years resulting in less severely affected dogs being treated in first opinion practice.

Laboratory assessment of coagulation parameters in this study did not include analysis of platelet count and function. Platelet counts were only submitted in 21 dogs; this reflects that fact that many of these dogs would have been admitted out of hours or at weekends and would therefore have had in house blood smear evaluation performed. In 18 dogs estimated platelet counts were available although in 8 of these cases a minimum count was provided due to the presence of clumps in the sample, resulting in a pseudothrombocytopenia. In a further 3

samples no count was provided, only an estimation of adequate numbers. It is likely therefore that the median platelet count presented here is an underestimation, however mild thrombocytopenia may also be a feature of this disease as has been previously reported (Cury *et al* 2002). Although it would have been preferable to have more accurate platelet estimates in a higher proportion of patients, it also seems unlikely that thrombocytopenia is the cause of coagulopathy in this disease as most dogs had a platelet count above that at which spontaneous haemorrhage is normally seen.

A large proportion of the study population had signs of bleeding identified on physical examination or at post-mortem. It is possible that some dogs included in the non-bleeding group had internal haemorrhage with no signs of external haemorrhage which may have introduced bias. This is particularly true of the dogs presenting with neurological signs where bleeding has been identified as the cause of signs in a number of cases (Garosi *et al* 2005, Wessmann *et al* 2006) Inclusion in this way however allowed us to assess the dogs as set out in our objectives.

Coagulation abnormalities were common in our population, with most dogs having one or more abnormality present, however there was no typical pattern. D-dimer concentrations were increased in 88% of dogs. Increased d-dimer concentration is associated with increased fibrinolysis as can be seen in systemic inflammation, neoplasia and following surgery. It is also present in cases of disseminated intravascular coagulation (Stokol *et al* 2000). The changes in d-dimer concentrations seen in this study may be a result of systemic inflammation or as a result of DIC. DIC is a complex coagulopathy associated with severe underlying diseases. It has features of both hypercoagulability and hypocoagulability resulting in both thrombosis and bleeding. DIC is a purported mechanism for the coagulopathy of *A.vasorum* (Wiinberg *et al* 2008 and 2010) and would explain the changes seen in fibrinogen concentration in this study. Fibrinogen concentration was significantly lower, and outside of the reference interval, in dogs that had signs of bleeding compared with those that did not. The diagnosis of DIC has not been standardized in dogs, although there is a model based scoring system (Wiinberg *et al* 2010). Application of this scoring

system was not possible in this study as it used specific ranges for tests run at the authors' laboratory. Our findings are suggestive of a consumptive process with activation of coagulation, consistent with DIC. It is not clear however whether these changes are the cause of the haemorrhage or a result of it.

Increases in fibrinogen concentration were seen in 9 dogs; hyperfibrinogenaemia has not been previously reported. It is likely related to the significant inflammatory response that occurs to the parasite in the pulmonary parenchyma (Caruso & Prestwood 1988).

Routinely performed coagulation tests were abnormal in 40% of cases, most of these dogs had signs of bleeding, however a further 8 dogs with signs of bleeding had normal coagulation tests. Hypocoagulability was identified on TEG in 22 dogs and 17 out of the 18 bleeding dogs had hypocoagulable TEGs. As PT and aPTT were normal in some cases it could be assumed that secondary coagulation is intact in some dogs bleeding with *A.vasorum* and supports a role for abnormalities in primary coagulation, including platelet function. Platelet dysfunction is difficult to evaluate using TEG, as it represents a global evaluation of coagulation. Platelet dysfunction, however, tends to be associated with an increased k and reduced MA on TEG, although this is not specific (Bowbrick *et al* 2003). Both of these were present in this population of dogs. Platelet function analysis was not performed in this group of dogs and given the findings and the suggestion of dysfunction would provide a logical area for further investigation.

TEG did identify hypocoagulability in 2 dogs presenting with neurological disease with no other visible signs of bleeding. Although haemorrhage was not confirmed in these dogs it seems a reasonable explanation. Given these findings TEG does not seem to offer any clear advantage over physical examination for identification of haemorrhage, however, it may be beneficial in cases where bleeding is the suspected pathogenesis and other tests have failed to identify a cause.

Identification of hypercoagulability is a useful application of TEG and in this series 3 dogs with hypercoagulability were identified. In 2 of these dogs

pulmonary hypertension was present, which was attributed to hypoxia and infiltration associated with angiostrongylosis. Pulmonary hypertension was not identified in any other dogs in this study, however echocardiography was not performed and therefore the incidence of pulmonary hypertension in this population is unknown. No other dogs showed clinical signs attributable to right sided heart failure. The relationship between hypercoagulability and pulmonary hypertension associated with angiostrongylosis would be an interesting area for further investigation as it may provide future therapeutic options.

This study provides some interesting results, but does not completely explain what is happening in dogs with bleeding signs secondary to angiostrongylosis. Dogs presenting with signs of haemorrhage have hypocoagulability present on TEG, but inconsistently have alterations in secondary coagulation. D-dimer concentrations are increased and may hint at the presence of DIC. In particular the findings suggest alterations in primary haemostasis. Further analysis of platelet function should be prioritized as an area of research in these dogsTEG may be useful in association with PT and aPTT in dogs presenting with bleeding in order to decide on the best use of blood products. In the presence of clinically relevant haemorrhage alterations in PT and aPTT would provide an indication for the use of fresh frozen plasma, whereas if these were normal with hypocoagulability on TEG primary haemostatic dysfunction may be suspected and other therapies may be preferred. While TEG provides the clinician with a method of globally evaluating coagulation it does not specifically evaluate platelet function and other methodologies may be more suitable for this such as multiple electrode aggregometry or advanced whole clot analysis. Because of its high sensitivity for identification of coagulopathy in this population TEG also be used to rule out significant coagulopathies. Although in bleeding dogs it does not seem to confer benefits in clinical practice over careful and thorough clinical examination or clinical suspicion of haemorrhage use of TEG may be particularly beneficial for identification of hypercoagulability and may influence therapy in this group in particular.

[A]SPSS Statistics 21, IBM, Hampshire, UK

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Table One

Fibrinogen concentration, antithrombin percentage activity, d-dimer concentration and von Willebrand factor concentration in dogs that had bleeding diatheses and those that didn't

Coagulation parameter	Not bleeding n=10 Median (IQ range)	Bleeding n=15 Median (IQ range)	P value
Fibrinogen (mg/dL)	545 (402)	108 (443)	0.026
Antithrombin (%)	87.5 (24)	90 (25)	0.935
d-dimer (ng/ml)	606.5 (533)	701 (661)	0.461
Von Willebrand factor (%)	185 (103)	110 (93)	0.129

Table two

TEG parameters in animals that were bleeding compared with those without evidence of bleeding

TEG parameter	Not bleeding n-12 median (IQ Range)	Bleeding (s) n=18(median)	p value
R (minutes)	10.4 (5.9)	17.5(9.7)	0.008
K (minutes)	3.3 (3.0)	N/A	N/A
A (angle)	40.0 (61.2)	10.0 (21.0)	0.001

MA (mm)	55.5 (13.1)	18.6 (30.0)	0.004
G dynes/s	6.2(3.1)	1.2 (2.8)	0.004

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387 Table three

388 Fibrinogen, antithrombin, D-dimers and von Willebrand factor concentrations in

389 dogs that were hypocoagulable on TEG and those that weren't

Parameter	Hypocoagulable n=18 Median (IQR)	Not hypocoagulable n-7 Median (IQR)	p value
Fibrinogen (mmol/l)	4·09 (1·20–17·43)	15·99 (13·11–22·76)	0.097
Antithrombin (%)	89·0 (75·25–102·00)	90·0 (80·00–98·00)	0.745
D-dimers (nmol/l)	3·74 (1·86–5·35)	2·86 (1·63–4·44)	0.574
Von Willebrand factor (%)	117.5 (145)	176.0 (69)	0.357

390